BARD Final Report IS-4937-16

Molecular dissection of host and pathogen factors in *Botrytis cinerea* pathogenesis for improved genetic resistance

Sharon, A. Tel Aviv University
Mengiste, T.D. Purdue University

Project award year: 2016
Three year research project

Abstract

Necrosis inducing proteins (NIPs) are cell death inducing effectors produced by necrotrophic pathogens. In this study we isolated and characterized NIPs from the plant pathogenic fungus *Botrytis cinerea.* Our objectives were to Identify and characterize novel *B. cinerea* NIPs and their plant targets. First, we collected fungal secretome from infected leaves and found that purified secretome has strong necrosis inducing activity as early as 22h post infection. In search of candidate NIPs, secretome was collected from bean leaves that were infected with wild type and pathogenicity mutants, proteins were partially purified and then subjected to proteomic analysis. Comparative analysis of secreted proteins in the wild type and pathogenicity mutants was used to select proteins that might be associated with pathogenic development. Candidate proteins were screened by Agrobacterium-mediated transient expression (agroinfiltration), and three candidates were identified. Two of these candidates, BcXyg1 and BcCrh1, were further characterized.

BcXyg1 is a GH12 xyloglucanase (BcXyg1) that induces strong necrosis and a resistance response in dicot plants. Analysis of disease dynamics showed that a *B. cinerea* strain over expressing *bcxyg1* produced early local necrosis supporting a role of BcXyg1 as an early cell death-inducing virulence factor. The xyloglucanase activity of BcXyg1 was not necessary for induction of necrosis and plant resistance, as a mutant protein lacking the xyloglucanase enzymatic activity retained both functions. Residues in two exposed loops on the surface of BcXyg1 were found necessary for induction of cell death, but not for induced plant resistance. Further analyses showed that BcXyg1 is apoplastic and interacts with the plant cell membrane, and that the BcXyg1-cell death-promoting signal is mediated by the LRR receptor-like kinases BAK1 and SOBIR1.

BcCrh1 is a GH16 transglycosidase that induces strong necrosis in several dicot plants, but not in Arabidopsis and monocots. Crh proteins catalyze crosslinking of chitin and glucan polymers in the fugal cell wall. We revealed a novel and unexpected role of *Botrytis cinerea* BcCrh1 as a cytoplasmic effector and elicitor of plant defense. During saprophytic growth the BcCrh1 protein is localized in fungal vacuoles and ER. Upon plant infection the protein accumulates to high levels in infection cushions, it is then secreted to the apoplast and translocated into plant cells, where it induces cell death and defense responses. Dimerization of BcCrh proteins was necessary for the transglycosylation activity and proper fungal development, while the monomeric proteins was sufficient for induction of cell death. *Arabidopsis* lines expressing the *bccrh1* gene had reduced sensitivity to *B. cinerea,* demonstrating the potential use of the protein in plant immunization against necrotrophic pathogens.

To shed light on plant factors that regulate NIP mediated defense elicitation we focused on Receptor like kinases (RLKs) and receptor-like cytoplasmic kinases (RLCKs). RLKs and RLCKs play crucial roles in early stages of signaling as components of receptor complexes and intracellular signal amplifiers, but their role in the recognition and signaling of elicitors derived from necrotrophic fungi such as *B. cinerea* have not been well elucidated. Three of the RLCKs and an RNAi lines in one of the RLCKs showed enhanced susceptibility to *B. cine*rea relative to wild type tomato plants. Similarly, we have isolated at least one RLK that shows enhanced susceptibility to *B. cinerea* and the *B. cinerea* NIPs. One of these RLCKs, TRK1, and its upstream and downstream components have been studied in detail and has been submitted for publication.

Collectively, our findings support the dual and opposite roles of NIPs as 1) virulence factor that facilitate infection of necrotrophic pathogens, and 2) elicitors of plant defense, which suppresses infection. Two types of NIPs have been described, BcXyg1, which is apoplastic, and BcCrh1, which is cytoplasmic. Both proteins were used to confer plant resistance, demonstrating the potential use of NIPs in plant immunization against necrotrophic pathogens.

Summary Sheet

Publication Summary

PubType	IS only	Joint	US only
Reviewed	1	1	1
Submitted	0	1	0

Training Summary

Trainee Type	Last Name	First Name	Institution	Country
Postdoctoral Fellow	Zhu	Wenjun	TAU	Israel
Postdoctoral Fellow	Maria Scalschi	Loredana	TAU	Israel
Postdoctoral Fellow	Jaiswal	Namrata	Purdue	USA
Postdoctoral Fellow	Bi	Kai	TAU	Israel
Postdoctoral Fellow	Frid	Renana	Tel Aviv University	Israel

Collaboration

The collaboration between the two labs was absolutely essential for the successful completion of the project. The two labs have complementary skills and expertise; the lab of the US PI works on plant molecular genetics and specializes in analysis of signaling cascades that mediate responses to pathogens. They apply genetic and biochemical approaches to isolate and study specific components of the plant immune system, in particular RLKs/RLCKs. The lab of the Israeli PI studies fungal plant pathogens and has specialized in the study of Botrytis virulence factors. This combination of skills allowed use to dissect the interaction from the plant and fungal sides. The work involved heavy exchange of materials and technologies, for example fungal strains and clones of fungal genes that were produced in Israel were shared with the US lab for transcriptiomic analyses, and pool down experiments that were conducted in Israel were guided by the US lab. The project has yielded three publications, two of which are co-authored by both PIs, which indicate the close and productive collaboration.

Major achievements

Botrytis cinerea is a devastating plant pathogen. This fungus causes grey mold and rot diseases in a wide range of agriculturally and economically important plants, and is the most significant pathogen in major crops including strawberry, tomato, grapes and cannabis. In addition to the damage to plants in the field, the fungus also causes rots in soft fruits and vegetables post-harvest and in storage. There are no good control measures against *B. cinerea* and therefore despite heavy use of chemicals, yield losses remain very high, exceeding $10 billion annually. New discoveries show that *B. cinerea* uses a range of mechanisms to overcome the plant defense to establish infection. These discoveries suggest that improved control strategies might be developed by boosting the appropriate components of the plants' defense. Still, there is a gap of knowledge on the specific players that control plant-fungus interplay and on their mode of action. The goal of this proposal was to help in closing the knowledge gap towards development of novel and chemical-free disease control measures. Specifically, we isolated and analysed necrosis-inducing proteins (NIPs), which are used by the pathogen to kill host cells, but at the same time are recognized by the plant immune system and activate defense responses that inhibit disease development and spreading.

To identify novel NIPs, an in vivo screening system was developed in which detached bean leaves were infected with *B. cinerea* spore suspension, the suspension (secretome) was collected at different time point post inoculation, cleaned, necrosis inducing activity was tested, and the protein content was determined by proteomic analysis. This work retrieved 259 proteins that were categorized according to biological function and other criteria. By comparison of secretomes from the wild type and fungal pathogenicity mutants we reduce the number of suspected NIP candidates, and then tested the necrosis-inducing activity of candidate proteins by a leaf assay. We found three NIPs and performed detailed analysis of two of them: BcXyg1, and BcCrh1. Both of these proteins are glycosyl hydrolases (GH), but they have different biological activities. BcXyg1 degrades hemicellulose (xylene), which is a structural sugar polymer of the plant cell wall, whereas BcCrh1 is involved in biosynthesis of the fungal cell wall. Our analyses showed that both proteins contribute to disease development, however they do it in different ways. Moreover, both proteins are recognized by the plant immune system and activate plant defense responses. Using protein manipulations, we produced protein derivatives that activate plant defense but don’t induce necrosis (cell death). Treatment of plants or expression in transgenic plants of the protein derivatives enhanced the plant defense response and reduced sensitivity of the plants to infection by Botrytis.

To shed light on the role of NIPs in defense activation, we studied plant factors that regulated NIP mediated defense elicitation. Receptor like kinases (RLKs) and receptor-like cytoplasmic kinases (RLCKs) play crucial roles in early stages of signaling as components of receptor complexes and intracellular signal amplifiers. RLKs associate with RLCKs and function as part of multi-protein immune complexes. The role of plant RLKs and RLCKs in the recognition and signaling of elicitors derived from fungi such as *B. cinerea* have not been well elucidated; neither the specific pathogen derived molecules nor the host components from perception to signaling are clear. In search of RLKs/RLCKs that might associate with the two NIPS, multiple RLCKs and RLKs were examined. Subsequently, we generated mutant plants and tested their response to infection by *B. cinerea*. We identified and isolated four RLCKs and one RLK that showed enhanced susceptibility to *B. cine*rea and the two NIPs. One of these RLCKs, TRK1, and its upstream and downstream components have been studied in detail.

Collectively, our results provided new knowledge and novel tools for rational-based manipulation of the plant defense, toward production of Botrytis resistant plants. One direction is utilization of the NIPs or parts of them to activate the plant defense responses. This can be done either by external treatment (spraying) with protein derivatives or by generation of transgenic plants that express these proteins. Another direction is to modify the plant proteins (RLKs/RLCKs) that mediate response to the NIPs. This can be done through gene editing (using CRISPR technology) as well as genetic engineering technologies. Development of such approaches will provide new control measures against Botrytis, and reduce the use of chemical fungicides, which contaminate the environment and are also not very effective against Botrytis due to development of fungal resistance.

Publications for Project IS-4937-16

Status	Type	Authors	Title	Journal	Vol:pg Year	Coun
Published	Reviewed	*Bi K, Scalschi L, Jaiswal GN, Frid R, Zhu W, Masrati G, Mengiste T, Sharon A*	The Botrytis cinerea Crh transglycosylae is a cytoplasmic effector triggering plant cell death and defense response	***BIORXIV***	2020 : 2020	Joint
Submitted	Reviewed	*Namrata Jaiswal, Chao-Jan Liao, Bemnet Mengesha, Han Han, Sang Yeol Lee, Sanghun Lee, Amir Sharon, Yun Zhou, Tesfaye Mengiste*	Regulation of plant immunity and growth by tomato receptor-like cytoplasmic kinase		: 2020	Joint
Published	Reviewed	*Wenjun Zhu, Mordechi Ronen, Yonatan Gur, Anna Minz-Dub, Gal Masrati, Nir Ben-Tal, Alon Savidor, Itai Sharon, Elad Eizner, Oliver Valerius, Gerhard H. Braus, Kyle Bowler, Maor Bar-Peled, Amir Sharon*	BcXYG1, a Secreted Xyloglucanase from Botrytis cinerea, Triggers Both Cell Death and Plant Immune Responses	***Plant Physiology***	175 : 438–456 2017	IS only
Accepted	Reviewed	*Siming Xu, Chao-Jan Liao, Namrata Jaiswal, Sanghun Lee, Dae Jin Yun, Sang Yeol Lee, Michael Garvey, Ian Kaplan, Tesfaye Mengiste*	Tomato PEPR1 ORTHOLOGUE RECEPTOR-LIKE KINASE1 regulates responses to systemin, necrotrophic fungi and insect herbivory	***The Plant Cell***	30 : 2214–2229 2018	US only

Summary of main findings

- **Identification of novel *B. cinerea* NIPs**. An in vivo screening system was developed in which detached bean leaves were infected with *B. cinerea* spore suspension, the suspension (secretome) was collected at different time point post inoculation, cleaned, necrosis inducing activity was tested, and amount of proteins were evaluated by western blot analysis. Using this system, we determined the onset of necrosis induction (about 18hpi), determined suitable time (28hpi) for secretome collection and conducted proteomic analysis of secretomes from wild type- and pathogenicity mutants-infected leaves. The results of this part have been published and are described in Zhu et al., 2017. In short: a total of 259 proteins were identified in the wild type secretome. The largest groups included enzymes of carbohydrate hydrolysis, proteins of unknown function, cell wall degrading enzymes, oxidoreductase, and proteases. The proteins were prioritized by comparison of wild type and mutant protein profiles and according to size, relative abundance, presence of secreted signal, protein characteristics, and GO annotation. About 40 protein candidates were cloned and screened by agroinfiltration of *N. benthamiana* leaves. Three necrosis-inducing candidates were identified: BcXyg1, BcCrh1, clone 144. Detailed analysis was conducted with BcXyg1 and BcCrh1.
- **Characterization of BcXYG1**. The *B. cinerea BC1G_00594* gene is a single copy gene, consisting of four exons and three introns. The first 18 N terminal amino acids encode a signal peptide and the entire predicted protein includes 248 amino acids. It encodes for a protein that we named BcXyg1 that is a GH12 xyloglucanase. BLAST searches of fungal genomes with BC1G_00594 showed the presence of homologs in a large number of necrotrophic and hemibiotrophic plant pathogens, but not in biotrophic plant pathogens. We cloned the gene and conducted a robust analysis of its structure and function. Results have been described by Zhu el al., 2017. In short: BcXYG1 showed strong necrosis-inducing activity in dicot plants, but not in monocot plants, and the cell death inducing activity was independent of the xyloglucanase catalytic activity. In a series of experiments, we found that BcXyg1 is an apoplastic effector and that the necrosis induction is mediated by the SOBIR-BAK1 complex probably through interaction with a yet unidentified RLP. The *bcxyg1* gene was highly expressed in planta, as oppose to moderate stable expression in culture conditions. Phenotypic analysis of fungal mutants showed no effect on colony growth, Morphology and stress tolerance, as well as on lesion size. However closer analysis of disease dynamics of *bcxyg1* over expression strain revealed earlier production of local necrosis during the initial disease phase. Treatment of plants with BcXyg1 induced defense responses, which were unrelated to induction of necrosis. Two epitopes of the protein were

found necessary for induction of necrosis, but not defense responses, which demonstrates the potential use of BcXyg1 in engineering plant resistance.

- **Characterization of BcCrh1**. Bcin01g06010 induced strong cell death in *N. benthamiana.* SMART analysis revealed presence of a conserved glycosyl hydrolase family 16 (GH16) domain, and BLAST search revealed similarity to proteins in the Crh family. The protein was named BcCrh1 based on the homology to the *Saccharomyces cerevisiae* Crh1 protein. We cloned the gene and conducted a robust analysis of its structure and function. Results are described by Bi et al (manuscript under review). In short: BcCrh1 triggered local cell death in *N. benthamiana* and tomato leaves, but not in Arabidopsis and monocot plant species. Induction of cell death was found unrelated to the enzymatic activity, and agroinfiltration assay of *N. benthamiana* leaves with different constructs and treatment with purified protein showed localization of BcCrh1 inside plant cells was necessary for induced cell death. Further studies confirmed uptake of BcCrh1 by the plant cells and revealed a 53 amino acid sequence at the N' end of the protein that is necessary and sufficient for protein uptake in a pathogen-independent manner. A series of deletion analysis were conducted in order to define the cell death-inducing region revealed that a peptide of 35 amino acids between residues 93-127 was necessary and sufficient for the induction of cell death. Infiltration of *N. benthamiana* and tomato leaves with Agrobacterium or purified protein solution resulted in accumulation of reactive oxygen species (ROS) and activation PTI genes, and when plants were inoculated with *B. cinerea* 48 h after infiltration with the protein, infection was significantly reduced. Further studies of gene expression showed that BcCrh1 is recognized by the plant as PAMP, and triggers a defense response, which is independent of the GH enzymatic activity and accompanied by development of necrosis. Since BcCrh1 does not induce cell death in *Arabidopsis thaliana*, we produced *A. thaliana* transgenic lines that express the BcCrh1 protein and tested their sensitivity to infection by the fungus. The BcCrh1 expressing plants were much less sensitive to infection by *B. cinerea* compared with control plants, showing that BcCrh1 induces PTI reaction, which can be used to improve plant resistance to *B. cinerea*. Analysis of fungal development during interaction with plants showed that Transcript levels of *bccrh1* increased sharply following infection and reached a peak 12 hpi. Further studies using a transgenic *B. cinerea* strain that expressed a *bccrh1-gfp* fusion gene showed that during saprophytic growth the gene is expressed constantly at moderate levels and the protein is localized inside the fungal cells, while in planta the gene is highly and transiently expressed following first contact of the fungus with the plant, and the protein accumulates to high levels in infection structures before being released to the plant apoplast. Analysis of transgenic fungal strains in which the *bcbcrh1* gene was manipulated in various ways led to discovery that BcCrh1 forms dimers, which are necessary for the cell wall related GH activity. Defects in the GH activity resulted in defected infection cushion and loss of pathogenicity. The specific accumulation of BcCrh1 in infection cushions implies that it is necessary for infection cushion formation, which is associated with retardation of hyphal extension and massive branching. Possibly, following these pathogenicity-specific developmental events, the excessive enzyme, now serving as a virulent factor, is released from the mature infection cushions and induce plant cell death.

- **Clone 01444**. This is a 90 amino acids protein without any known domain and structures. The protein contains 10 cysteine residues that are predicted to form 5 disulfide bonds. As such, it is a classical effector protein. Blast analysis showed that homologs of 1444 are found only the closely related pathogen *Sclerotinia sclerotiorum* and another necrotrophic pathogen *Alternaria alternate*, but it is not found in a wide range of other pathogenic fungi. *Agrobacterium tumefaciens* infiltration assay of *N. benthamiana* leaves with PVX (Potato virus X) plasmid pGR106 induced obvious chlorosis in the infiltrated leaf and stunting of the rest of the plant. The upper untreated leaves also shown spotted-like chlorosis 20 days after infiltration, indicated that 01444 moved to upper new leaves, induce chlorosis and inhibit the growth of the plant. The gene is highly expressed at late stages of infection, supporting the possibility that 1444 is necessary for pathogenicity at the stage of lesion expansion. In the next year we will continue the analysis of this protein and its role in pathogenicity.
- **Identification of plant RLKs/RLCKs that mediate Botrytis infection**

  To identify genotypes and variants with altered responses to BcXyg1, we have screened several cultivars of cultivated tomato, wild species related to tomato with purified BcXyg1 proteins for necrosis. We did not see clear difference in different wild Solanum species related to the cultivated species. However, we observed enhanced necrosis on tomato fab2 mutant which is defective in the receptor like kinase (RLKs). In addition, we identified a tomato receptor like cytoplasmic kinase RLCK due to its altered responses to infiltration by this fungal protein. The tomato RLK is the receptor for the growth peptide CLVATA3 while RLCK is a recently identified tomato protein kinase that shows enhanced susceptibility to *B. cinerea*. At low concentration (100ng) of BcXyg1, both the RLK and RLCK mutant plants showed enhanced necrosis earlier than their wild type plants. We also tested the expression of defense gene PR1 following infiltration of WT tomato leaves with GFP or the B-364 proteins. We observed strong induction of PR1 gene expression at 24 h after infiltration.

- **Transcription response of tomato plants to BcXyg1 and BcCrh1**

  Genome wide gene expression profile of tomato plants treated with BcXyg1 and BcCrh1 were conducted through RNA-seq. Genes with induced or reduced gene expression in response to the NIPs were identified. The cellular processes and biochemical pathways that are induced or suppressed by the two NIPs were deciphered from that analyses. Some of these selected genes will be selected for further studies.